Time-Lapse Confocal Imaging of Development of *Bacillus anthracis* in Macrophages

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Macrophages attempt to battle infection with *Bacillus anthracis* spores by phagocytosis of the spores. However, it is believed that *B. anthracis* spores may survive phagocytosis and may actually use the macrophages that ingest them as a means of transport to lymph nodes. Thus far, the events that occur after spores undergo phagocytosis have remained unclear. To elucidate the fate of spores internalized by macrophages, we have used time-lapse confocal microscopy to follow individual fluorescent spores over time. By use of this method, we have determined that some phagocytized spores survive beyond germination, to become bacilli that then replicate within the macrophages.

The interaction between *Bacillus anthracis* spores and macrophages is a pivotal link in the progression of the disease anthrax, which has become an urgent topic of research because of the previous use of *B. anthracis* as a biological weapon and the fear that it may be used again. Inhaled spores hijack macrophages [1], which normally destroy invading organisms, for transport from the inhospitable environment in the alveoli of the lungs to the more-favorable growth environment of lymph nodes [2, 3]. Previous attempts to chronicle the interaction between spores and macrophages by use of microscopy have been hindered by the limitation of "snapshot" views of fixed specimens, leading to conflicting results [4–6]. We present, here, the first report to track the life histories of individual fluorescent spores and show that phagocytized spores can develop into bacilli and replicate within macrophages before ultimately killing the macrophages.

Methods. The Sterne strain of *B. anthracis* (an attenuated strain that does not produce capsule) was transformed with a plasmid that expresses green fluorescent protein (GFP). GFP is not linked to any other protein, is abundantly present in the interior of the bacillus and of the spore (as determined by anti-GFP immunogold-electron microscopy), and does not affect colony size, colony shape, or bacterial doubling time, compared with the parent Sterne strain (data not shown). A detailed description of the method used and the resulting fluorescent *B. anthracis* is currently in preparation, but a brief account is provided below. Plasmid pAFp8gfp is a derivative of pUB110, which confers kanamycin resistance to gram-positive strains harboring it. The sequence of a synthetic promoter was derived from analysis of a number of potential highly expressed *B. anthracis* genes by examining the 5′ flanking sequences of homologues to potential highly expressed *Bacillus subtilis* genes [7]. A small DNA fragment containing the synthetic promoter was placed just upstream of the *pag* gene, on a shuttle vector previously constructed by ligation of a pPA102 [8] derivative, in which *gfp* had been placed downstream of *pag*, to pBR322. Transformation of the resulting plasmid into *B. subtilis* strain WB600 was selected by culturing on Luria-Bertani agar plates supplemented with kanamycin (10 μg/mL). A spontaneous deletion of *pag* (and pBR322), in which *gfp* was expressed at noticeably higher levels, was obtained. Subsequent studies have shown that pAFp8gfp can be transformed into various strains of *B. anthracis*, is stable, and confers constitutive synthesis of GFP. The spores produced by the transformed *B. anthracis*, which were likewise fluorescent and, therefore, easily and immediately identifiable, were used to infect RAW264.7 macrophage cell cultures stained with 25 μmol/L $DiIC_{16}(3)$, a fluorescent lipophilic dye that stains cellular membranes (Molecular Probes). We found that DiI predominantly stained internal membranes and, therefore, did not mark the full extent of the cells but was sufficient as a counterstain to determine whether spores were intracellular. Spores were harvested from shaking-broth cultures of Leighton and Doi medium, were purified by centrifugation with 58% Hypaque-76 (Amersham Health), and were resuspended in sterile water. Before infections of macrophages, a 1×10^8 cfu/mL dilution of spores was heat-shocked for 45 min at 60°C to ensure that none of the spores used for infection had already germinated. RAW 264.7 cells (ATCC TIB 71) were maintained at 37°C in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco BRL) and, 1 day before infection, were subcultured in 6-well tissue-culture plates containing 25-mm glass coverslips. Spores were diluted into DMEM/10% FBS and added to

Received 10 July 2003; accepted 2 October 2003; electronically published 19 March 2004.
Presented in part: 5th International Conference on Anthrax, Nice, France, 31 March 2003 (abstract P210).

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The Journal of Infectious Diseases 2004; 189:1313–6

0022-1899/2004/18907-0024

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 APR 2004	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Time-lapse confocal imaging of development of Bacillus anthracis in macrophages. Journal of Infectious Diseases 189:1313 - 1316	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
Ruthel, G Ribot, WJ Bavari, S Hoover, TA	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

The original document contains color images.

14. ABSTRACT

Macrophages attempt to battle infection with Bacillus anthracis spores by phagocytosis of the spores. However, it is believed that B. anthracis spores may survive phagocytosis and may actually use the macrophages that ingest them as a means of transport to lymph nodes. Thus far, the events that occur after spores undergo phagocytosis have remained unclear. To elucidate the fate of spores internalized by macrophages, we have used time-lapse confocal microscopy to follow individual fluorescent spores over time. By use of this method, we have determined that some phagocytized spores survive beyond germination, to become bacilli that then replicate within the macrophages.

15. SUBJECT TERMS

Bacillus anthracis, anthrax, development, macrophages, confocal microscopy

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**4**	

the monolayer at a concentration that resulted in an average MOI of 4 spores/macrophage (range, 1–11 spores/macrophage). After the addition of spores to the macrophage cultures, culture plates were spun for 5 min in a centrifuge at 52 *g* to quickly bring spores into contact with the macrophages and to synchronize infection. Cells were incubated with the spores for 15–20 min and then were rinsed several times with DMEM. To confirm that spores had been phagocytized, we maintained the infected cell cultures in a cell culture chamber on a Nikon TE300 microscope stage at 37°C and collected a minimum of 30 confocal optical sections at 0.5-μm intervals throughout the cells. Confocal images were collected at 30-min intervals for 7–8 h by use of a BioRad 2000MP confocal system (Hemel Hempstead). Spores were scored as being intracellular if they were entirely and unambiguously surrounded by the DiI fluorescence used to label the cells. To ensure that the macrophages were not dead before growth and that replication of the internalized spores occurred, we repeated these experiments in the presence of 1–2 μmol/L Sytox green, a DNA dye that does not cross the intact membranes of living cells and, therefore, acts as a dead-cell reporter. By carefully examining the series of optical planes collected by confocal microscopy, we were able to distinguish bacilli that arose from internalized spores from bacilli that had been phagocytized after extracellular development. This was not previously possible without the use of the antibiotic gentamicin, which eliminates extracellular bacteria [4] but can also halt growth of intracellular bacilli [5].

Results. Although the macrophages were apparently capable of killing many of the phagocytized spores, many germinated spores were observed to become vegetative and replicate intracellularly (movies of the sequences shown in figure 1 are available in the electronic edition of the *Journal* [http://www.journals.uchicago.edu/JID/journal/issues/v189n7/31432/31432.html]). Of 315 spores determined to have been internalized by macrophages (79 infected macrophages were observed), 34 (10.8%) were observed to become vegetative and replicate. Although timing varied, newly germinated bacilli typically began to noticeably elongate by 4–5 h and replicated by 5–6 h. This development was delayed by ~1–2 h, compared with that of extracellular spores, presumably due to the time required for escape from phagolysosomes. The shape of the macrophages typically reflected the growth of the bacilli within the macrophages; that is, the plasma membrane of macrophages stretched to accommodate the bacilli (figure 1*A*, brightfield images). In some cases, after ~7 h, we noted rupturing of macrophages containing particularly long chains of bacilli, which was obvious in transmitted-light images taken concurrently with the fluorescence images. Spores often grew into chains of bacteria, which is typical for *B. anthracis*, within cells before the death of those cells. The use of Sytox green as an indicator of membrane integrity confirmed what we had concluded on the

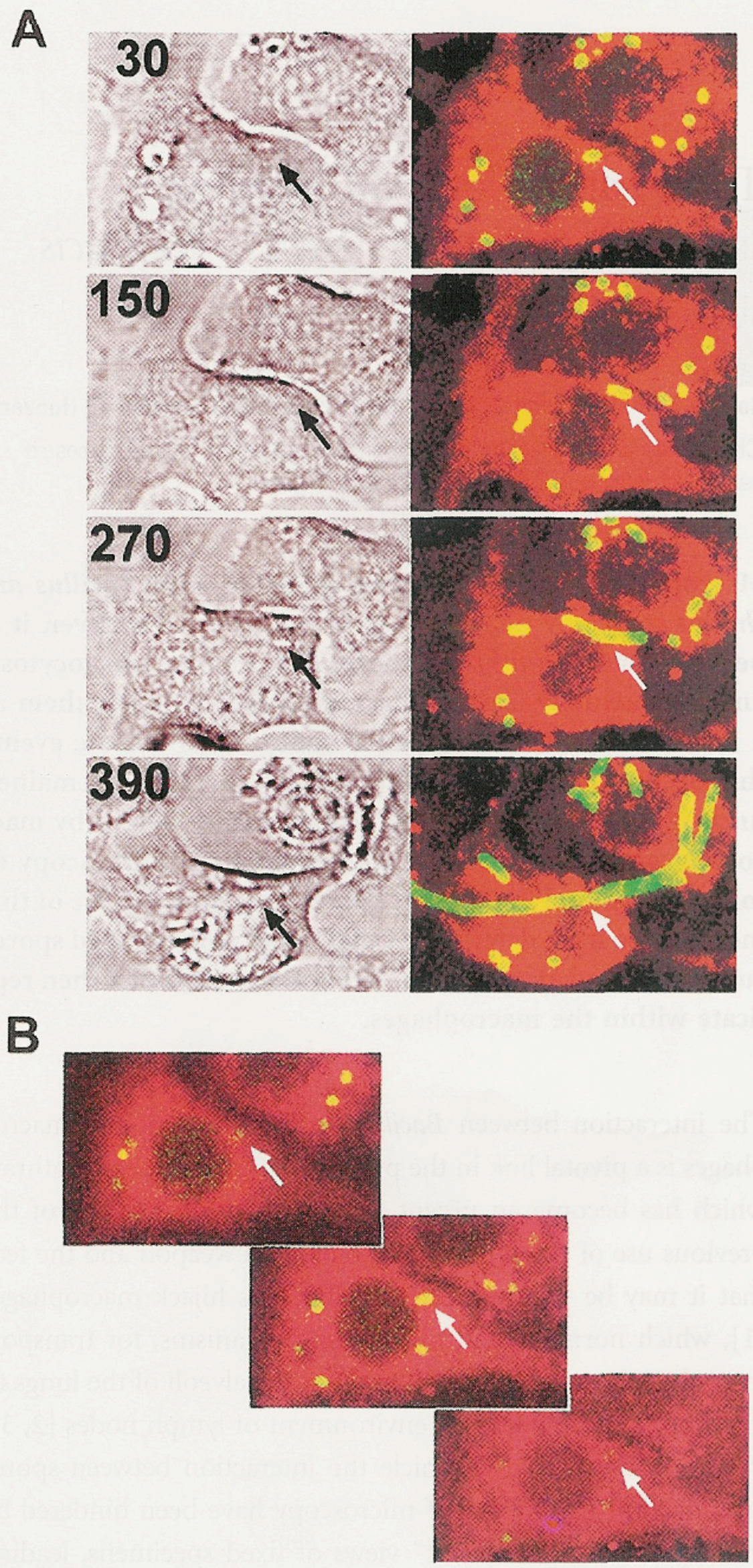

Figure 1. Growth and replication of *Bacillus anthracis* in RAW264.7 macrophages. *A*, Brightfield *(left)* and fluorescence *(right)* confocal micrographs of a representative $DiIC_{18}(3)$-stained macrophage *(red)* infected with green fluorescent protein–expressing spores *(green)*, at 4 time points. The time after the start of recording is shown in minutes. The fluorescent images are reconstructed from optical planes taken throughout the entire cell at 0.5-μm intervals. Growth and replication of bacilli that began as an internalized spore *(arrows)* are obvious by 270 min. *B*, The intracellular location of the spores that produced bacilli was verified by examination of the separate confocal optical planes that had been gathered. Shown are 3 of these planes from near the top, middle, and bottom of the cell (respectively), at 30 min. The width of the image field shown is 35 μm. These images are from video 1 and video 2, available in the electronic edition of the *Journal*.

basis of morphological criteria—bacterial replication occurred in living cells.

Since only a fraction of the phagocytized spores were found to produce replicating bacilli (the rest, presumably, were killed within the macrophages) and since not all infected macrophages contained replicating bacilli, the question arose whether the number of spores infecting a single macrophage may be a determining factor of whether the macrophage survives the spores or the spores survive the macrophage. Indeed, we found that the average number of spores per macrophage was significantly different between macrophages that later contained replicating bacteria (mean ± SEM, 5.95 ± 0.77) and those that did not (mean ± SEM, 3.38 ± 0.31) ($P = .003$, Mann-Whitney rank sum test). That an alveolar macrophage is capable of engulfing multiple spores after an aerosol infection was convincingly demonstrated by Sirard et al. [9]. This is also consistent with the rather large number of spores known to be required for fatal subcutaneous infection of mice (LD_{50}, 2.5×10^3–2.0×10^7 cfu of Sterne strain spores, depending on the strain of mouse) [10] and fatal aerosol infection of rhesus macaques (LD_{50}, 5.5×10^4 cfu of Ames strain spores) [11]. Taken together, these data suggest that higher MOI increase the chances that the spores will overcome the defenses of the macrophage and develop into replicating bacilli.

Discussion. One concern that we had with our experimental paradigm was that production of protective antigen (PA) and lethal factor (LF) proteins by bacilli growing outside of the macrophages might have affected the macrophages and, therefore, the outcome of the experiments. However, we repeated the experiments using a *B. anthracis* strain lacking the pX01 plasmid that encodes these toxins, in addition to lacking the pX02 plasmid necessary for producing capsule that is also absent in the Sterne strain, and were again able to observe spores that germinated, became vegetative, and replicated within macrophages (data not shown). Aside from confirming our previous results, these further experiments indicated that PA and LF proteins are not essential for the replication of *B. anthracis* within macrophages. Subtle differences, such as the length of time required before replication occurs, may exist without the pX01 plasmid, but it will require further study to determine this with certainty.

In previous studies, the examination of separate populations of cells at different time points could not rule out the possibility that chains of bacteria found within macrophages may have developed extracellularly and subsequently been phagocytized. In fact, we observed instances in which bacterial chains that had developed outside of the macrophages were engulfed (results not shown). In the present study, time-lapse confocal microscopy, beginning at the spore stage, allowed us to clearly differentiate replication within macrophages from phagocytosis of bacterial chains.

Studies in which replication of the bacteria in macrophages did not occur have used gentamicin in the cell culture medium to eliminate the concern of postreplication phagocytosis. Results of previous work by Dixon et al. [5], who used permeability assays, suggested that the pore-forming toxins produced by *B. anthracis* might make the macrophage membrane leaky, thereby allowing some entry of gentamicin into the cells. To determine whether the presence of gentamicin in the cell culture medium might interfere with the growth of intracellular bacilli, as has been previously suggested [5], we repeated the experiments using medium containing 2.5 μg/mL gentamicin, added 15–20 min after addition of the spores. Of 218 spores determined to have been internalized by macrophages (59 infected macrophages were observed), only 1 (0.5%) was observed to become vegetative and replicate (figure 2). The 1 chain of bacilli seen to form in a macrophage with gentamicin present in the culture medium also appeared to be killed (on the basis of a swollen shape and cessation of growth) after the macrophage later lysed. These results suggest that the addition of gentamicin may have influenced the outcomes of previous studies that concluded that phagocytized spores do not develop beyond germination.

We have used the method of time-lapse confocal microscopy with GFP-expressing spores of *B. anthracis* to show that phagocytized spores are capable of producing bacilli that replicate

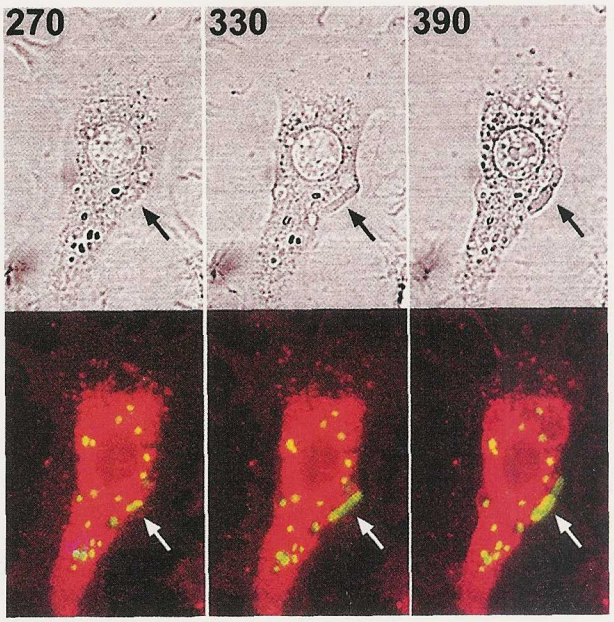

Figure 2. Infection of macrophages by *Bacillus anthracis* spores, with 2.5 μg/mL gentamicin added to the medium 20 min after addition of the spores. The time after the start of recording is shown in minutes. Only 1 spore was observed to produce replicating bacilli and is shown in this time series. After replication of the bacilli, the macrophage lyses (as apparent in the brightfield image above) and the bacilli cease their growth and appear to die quickly thereafter. The width of the image field shown is 40 μm.

within the macrophages. Furthermore, we have used this method to validate concerns raised against previous methods used to address the degree of development that *B. anthracis* spores can undergo inside the macrophages. Our finding is relevant to whole-animal infection by the aerosol route, since it is consistent with the hypothesis that spores can utilize macrophages or other phagocytic cells to travel from the lung to lymph nodes and there escape from those cells and spread throughout the host. Our results further suggest that the bacteria are capable of beginning replication even before they escape from the phagocytic cells.

Further study using the present method, to provide more-detailed histories of both the surviving and the nonsurviving subpopulations of spores, may yield a strategy to help macrophages overcome infection with *B. anthracis* spores. Experiments with various *B. anthracis* strains expressing GFP are also under way to determine the contribution of known virulence factors to this early stage of anthrax and to identify other currently unknown virulence factors.

Acknowledgment

We are grateful to Bob Curry for excellent technical assistance.

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